Relation Between High Energy Phosphate and Lethal Injury in Myocardial Ischemia in the Dog

Robert B. Jennings, MD, Hal K. Hawkins, MD, PhD, James E. Lowe, MD, Mary L. Hill, BS, Sherry Klotman, MS, and Keith A. Reimer, MD, PhD

The relationship between progressive depletion of high energy phosphate and the onset of lethal cell injury in ischemic myocardium following coronary occlusion has been evaluated. Myocardial ischemia was induced by proximal occlusion of the circumflex coronary artery for 15, 30, 40, or 60 minutes. Cell injury in the severely ischemic posterior papillary muscle (PP) was evaluated by electron microscopy and by measuring the capacity of slices of the injured PP to maintain electrolytes, resynthesize high energy phosphate, and exclude inulin during *in vitro* incubation. ATP content in the ischemic myocardium decreased to 35%, 9%, 7%, and 5% of control values after 15, 30, 40, and 60 minutes of ischemia, respectively, and was associated with a corresponding depletion of total adenine nucleotides. The loss of 65% of the ATP after 15 minutes of ischemia (reversible injury) was associated with only minimal ultrastructural changes and no significant defects of electrolytes in incubated slices. However, the depletion of over 90% of the ATP after 40 minutes of ischemia (irreversible injury) was associated with significant fine structural changes and markedly altered cell volume regulation. The results suggest a close relationship between the marked depletion of high energy phosphates and the development of lethal injury in acutely ischemic myocardium. (Am J Pathol 92:187–214, 1978)

SUDDEN OCCLUSION of a major coronary artery in the dog results in rapid conversion to anaerobic metabolism [1-7] with consequent loss of high energy phosphate from the ischemic myocardium. Initially, adenosine triphosphate (ATP) is replenished from creatine phosphate (CP) and the tissue content of the latter is reduced to 30% by 60 seconds of ischemia.[1,8]

The subsequent levels of ATP in the ischemic myocardium reflect the balance between utilization and production. Since the ischemic myocardium ceases to contract within 60 seconds of coronary occlusion,[9] the utilization of ATP for myosin ATPase is markedly reduced. Metabolic pathways which continue to utilize ATP under anaerobic conditions include reactions involved in the maintenance of ion distribution and cell volume,[10] enzyme reactions such as adenyl cyclase,[11] myokinase,[12] fatty acid synthetase,[13] some protein kinases,[14] and a variety of nonspecific

From the Department of Pathology, Duke University Medical School, Durham, North Carolina.

Supported in part by Grant HL18833 from the National Institutes of Health and by NIH Contract 1HV52999.

Accepted for publication March 14, 1978.

Address reprint requests to Robert B. Jennings, MD, Department of Pathology, Duke University Medical Center, Durham, NC 27710.

0002-9440/78/0710-0187$01.00

phosphatases. Synthesis of high energy phosphate continues in the ischemic tissue but at a much reduced rate compared with control aerobic conditions,[3,4,15,16] because anaerobic glycolysis is the only source of new high energy phosphate, producing 3 moles of ATP per mole of glucose derived from the breakdown of glycogen to lactate.[15] In severely ischemic myocardium, little plasma glucose is available and the amount of ATP which can be produced via glycolysis is limited by a) the cellular supply of glycogen,[16] b) the inhibition of glycolysis as the intracellular environment becomes more acid,[3,5] and c) the destruction of nucleosides and nucleotides during metabolism, eg, by 5′-nucleotidase, which cannot be resynthesized in anaerobic heart muscle.[12]

Several studies have evaluated the relationships of tissue high energy phosphate to the efficiency of contractile function [17-20] and to the capacity of myocardium to recover function after varying periods of ischemia, anoxia, or hypoxia.[5,21,22] The latter studies have shown that depletion of high energy phosphate is associated with failure of the cells to recover contractile function. However, failure to recover contractile function is not synonymous with cell death, and the relationship between high energy phosphate and lethal myocardial ischemic injury has not been explored. Accordingly, the present study was done to determine the relationship between ATP depletion and cell death as evaluated by characteristic changes in ultrastructure, cell volume regulation, and reperfusion studies. The results of these experiments suggest a close relationship between the level of high energy phosphate and lethal injury in acutely ischemic myocardium.

Materials and Methods

Twenty-one healthy dogs of either sex, which had been fasted overnight, were used.

Operative Technique

Dogs were anesthetized with intravenous pentobarbital. Respiration was maintained with a Harvard Model 607 respirator pump at a rate of 200 ml/min/kg with minor adjustments when necessary to achieve physiologic levels of blood P_{CO_2} and pH. Low-flow O_2 was provided at the air intake tube to keep the $P_{O_2} > 90$ mmHg. The left chest was opened through a small incision in the left fourth intercostal space. The circumflex branch of the left coronary artery was isolated under the left atrial appendage while the heart was temporarily supported in a pericardial cradle. Arterial blood pressure was measured through a catheter in the femoral artery connected to a Model P23D Statham pressure transducer. Limb lead II of the standard electrocardiogram (ECG) was recorded on a Brush Model 440 recorder. After hemodynamic equilibration, the artery was occluded by snaring it with a silk suture.

Experimental Design

Coronary occlusion was maintained for 15, 30, 40, or 60 minutes in groups of 4 to 6 dogs randomly selected at each time interval. Following the desired period of ischemia, the

heart was excised within 5 to 10 seconds and plunged into 750 ml of ice-cold isotonic KCl. After cooling for 2 to 3 minutes, the left ventricle was opened and subendocardial tissue was obtained from ischemic and nonischemic areas according to the techniques described below.

Identification of Areas of Severe Ischemia with Thioflavine S

Occlusion of the circumflex artery under these conditions produces a large area of ischemia on the posterolateral surface of the heart, which includes the posterior papillary (PP) muscle of the left ventricle. However, the apical portion of the PP in some dogs is supplied by the anterior descending coronary artery, and inclusion of such nonischemic tissue in samples of PP would affect assessment of changes. To avoid this possibility, the area of severe ischemia was identified by injection of the dye thioflavine S immediately prior to excision of the heart, as previously described.[23,24] Examination under ultraviolet light showed the region of severe ischemia as a nonfluorescent region surrounded by brightly fluorescent well-perfused tissue. Only nonfluorescent tissue was included in samples taken for electron microscopy and estimation of metabolites and cell volume.

The anterior papillary (AP) muscle of the left ventricle was used as nonischemic control tissue. Occasionally, the ischemic region extended into a portion of the AP. In such cases the nonfluorescent portion was excised from the AP prior to sampling.

In Vitro Slices

The severely ischemic PP and control AP tissues were trimmed from the heart and placed *en bloc* in ice-cold Krebs–Ringer–phosphate (KRP). From 20 to 35 thin free-hand slices were obtained first from the AP and then from the PP. Metabolites, cell volume, and ultrastructure were studied in those slices before and after incubation (as described below).[25,26]

For metabolite assays, the slices were weighed quickly on a Cahn Model DTL microbalance and placed in ice-cold 3.6% perchloric acid (PCA). Weighing and transfer to the PCA required 10 to 15 seconds. After 60 to 180 minutes, the slices were homogenized with a Tri-R homogenizer and neutralized with K_2CO_3 and KOH. The extracts were centrifuged to remove $KClO_4$ and the supernatant was frozen at −20 C. Samples were assayed within 2 to 3 weeks by enzymatic techniques for lactate,[27] ATP,[28] adenosine diphosphate (ADP),[29] adenosine monophosphate (AMP),[29] CP,[28] and glucose-6-phosphate (G6P).[28]

Assessment of Cell Volume and Electrolytes

Slices were incubated at 37 C in oxygenated KRP solution for 60 minutes. The KRP was prepared fresh, pH 7.2 to 7.4, and contained the following ions in millimoles per liter. Na^+, 151.6; K^+, 4.81; Ca^{2+}, 1.29; Mg^{2+}, 1.20; SO_4^{2-}, 1.24; PO_4, 15.63; and Cl^-, 121.7. Trace quantities of ^{14}C-hydroxymethyl-inulin were added to the incubating media [25] to determine the inulin-diffusible space (IDS).

As many as six slices weighing 20 to 80 mg wet weight were incubated in 15 ml of media at 37 C in a 25-ml Erlenmeyer flask. The medium was equilibrated with 100% oxygen prior to placing it into flasks, and the flasks were continuously gassed with 100% oxygen during incubation. All flasks were shaken 180 times per minute on a Dubnoff shaker.

Control slices of unincubated tissue were weighed quickly on a Cahn Model DTL electrobalance, placed directly into rinsed, ion-free scintillation vials (minivials, Corning), and dried at 105 C. The dry slices were reweighed and total tissue water (TTW) was calculated as milliliters per 100 g dry tissue. Incubated slices were rinsed by rapid dipping in 0.25 M sucrose (J. T. Baker), blotted quickly on Whatman filter paper, weighed, and dried as described above.

Electrolytes were extracted from each dried slice in 5 ml of 0.75N HNO_3.[30,31] The ions were determined in a 1 to 100 dilution of each HNO_3 extract prepared in ion-free

glassware verified to have no detectable sodium. RbCl, 5000 ppm, was added to each dilution and to the standard to equalize ionization between standards and unknowns. Electrolytes were measured against appropriately diluted standards in an IL 351 atomic absorption spectrophotometer interfaced with a Tektronix Model 31 computer calculator. Standard curves for Mg^{2+}, Na^{+}, and K^{+} were prepared from a standard solution containing these ions and PO_4 in physiologic concentrations.[31]

To calculate the IDS, the dry slices were rehydrated by adding one or two drops of deionized water to the vial. The tissue was solubilized in Soluene 350 and ^{14}C activity was counted in a Packard liquid scintillation counter.[25]

Electron Microscopy

For each of the four periods of ischemia, blocks from both the surface and center of at least two slices before and after incubation of both AP an PP muscles were cut under 4% glutaraldehyde in 0.1 M cacodylate buffer. After 1 to 4 hours of fixation, the blocks were postosmicated, dehydrated in a graded ethanol series rinsed in propylene oxide, and embedded in Epon 812.[32] Thin sections were cut from an average of three blocks per slice, stained with toluidine blue, and examined by light microscopy. Ultrathin sections were cut from at least two representative blocks of each slice, stained with uranyl acetate/lead citrate, and examined in a Hitachi Model HS-8 electron microscope.

Results

Coronary occlusion for 15 or 30 minutes caused no gross indication of injury, but gross evidence of injury was seen in 3 of 6 dogs by 40 minutes and in all dogs by 60 minutes. In the hearts of the latter group, the ischemic PP muscle was pale gray and stiffer than the nonischemic AP muscle. In all hearts, whether or not injury was visible grossly, the subendocardial region of most severe ischemia could be seen as a region of nonfluorescence under ultraviolet light. Nonischemic tissue, which had been perfused by Thioflavine-S injected intravenously just before excision of the heart, was brightly fluorescent.

Fine Structure

The ultrastructural appearances of myocytes in the center of freshly cut slices of nonischemic AP muscle or of PP muscle injured by various periods of ischemia were similar to those described previously.[33] The characteristic features of nonischemic myocardium are illustrated in Figure 1.

After 15 minutes of severe ischemia, myocytes of the PP muscle showed only minor differences from control tissue, including some margination of nuclear chromatin, and decreased amounts of glycogen (Figure 2). Myofibrils were relaxed and showed I bands.

After 30, 40, and 60 minutes of ischemia, the PP muscle cells showed progressively more extensive changes, with marked margination of nuclear chromatin, mitochondrial swelling, and mitochondrial amorphous matrix densities (Figure 3). The sarcolemma was flattened and showed

occasional tiny defects in the plasma membrane although the basal lamina remained intact. The changes became more severe as the period of ischemia was prolonged.

The effects of *in vitro* incubation for 60 minutes on slices injured by 40 or 60 minutes of *in vivo* ischemia are illustrated in Figures 4 and 5, respectively. The most striking change was the development of subsarcolemmal blebs which were associated with breaks in the plasma membrane of the sarcolemma. Remnants of the plasma membrane could be observed as circular profiles under the basal lamina, which often remained intact. Cells with membrane defects also developed prominent myofibrillar contraction bands.

Metabolite Changes

The ATP content of ischemic and nonischemic tissue was measured in 21 dogs (Table 1). The ATP of the control nonischemic AP was similar at all time intervals studied, and the data have been pooled. The ischemic PP tissue exhibited a progressive loss of ATP to 35%, 16%, 9%, and 7% of control levels after 15, 30, 40, and 60 minutes of ischemic injury, respectively. Severe ischemia also caused a rapid decrease in tissue CP. By 15 minutes, CP was 56% lower in the PP than in the AP; by 30 minutes or more, little CP remained. The lactate content of the PP muscle increased progressively as the period of ischemia was prolonged and was 400% more than that of the AP after only 30 minutes of ischemia.

Table 1—Metabolites of Normal and Ischemic Myocardium

Metabolite	Control AP (μmole/g wet weight)	PP (μmole/g wet weight) Minutes of ischemia 15	30	40	60
ATP	5.57 ± 0.19 (21)	1.94 ± 0.27* (5)	0.90 ± 0.15* (6)	0.50 ± 0.13* (6)	0.38 ± 0.14* (4)
CP	2.10 ± 0.13 (21)	1.18 ± 0.21† (5)	0.63 ± 0.16* (6)	0.31 ± 0.13* (6)	0.53 ± 0.14* (4)
G6P	1.24 ± 0.17 (21)	2.39 ± 0.15† (5)	1.49 ± 0.1† (6)	1.82 ± 0.5 (6)	1.74 ± 0.84 (4)
Lactate	9.21 ± 0.61 (18)	30.5 ± 4.5* (3)	40.9 ± 2.8* (5)	44.9 ± 2.4* (6)	—

AP = anterior papillary muscle, PP = posterior papillary muscle, CP = creatine phosphate, G6P = glucose-6-phosphate.

Mean differences between AP tissue and PP tissue were assessed after 15, 30, 40, or 60 minutes of ischemia. Statistical comparisons were made by a two-tailed non-paired *t* test, except for G6P, for which a paired *t* test was used.

* $P = < 0.001$.

† $P = < 0.01$.

The distribution of adenine nucleotides was measured in 12 of these dogs (Table 2). The progressive loss of ATP in the ischemic tissue was associated with a decrease in the total adenine nucleotide pool. The control tissue contained 7.41 ± 0.27 μmole of adenine nucleotides per gram wet weight. Of this total, 6.02 μmole was ATP, 1.23 μmole was ADP, and 0.16 μmole was AMP. After 15 minutes of ischemia, the total nucleotides were reduced by 55%, due chiefly to the loss of 3.9 μmole of ATP without a significant reciprocal increase in either ADP or AMP. In fact, there was a small but significant decrease in ADP. By 30 and 40 minutes there were further decreases in the total adenine nucleotide pool, again, chiefly due to the loss of ATP. The ADP content remained depressed. Although the AMP content continued to rise, reaching a level 631% greater than control after 40 minutes of ischemia, this rise accounted for

Table 2—Adenine Nucleotide Distribution of Normal and Ischemic Myocardium

Nucleotide	Control AP (μmole/g wet weight)	PP (μmole/g wet weight) Minutes of ischemia 15	30	40
ATP	6.02 ± 0.21 (12)	2.12 ± 0.45* (3)	0.82 ± 0.18* (5)	0.37 ± 0.07* (4)
Percent reduction in ATP content		65	85	94
ADP	1.23 ± 0.06 (11)	0.83 ± 0.01* (3)	0.96 ± 0.05† (5)	0.74 ± 0.09* (4)
Percent reduction in ADP content		33	22	40
AMP	0.16 ± 0.01 (11)	0.37 ± 0.11† (3)	0.66 ± 0.23† (5)	1.17 ± 0.19* (4)
Percent increase in AMP content		131	313	631
Total ATP + ADP + AMP	7.41 ± 0.27 (11)	3.32 ± 0.34* (3)	2.50 ± 0.15* (5)	2.28 ± 0.22* (4)
Percent decrease in total nucleotides		55	66	69
Adenylate charge ratio $\frac{ATP + 1/2\ ADP}{ATP + ADP + AMP}$	0.9	0.76†	0.54†	0.32†

AP = anterior papillary muscle, PP = posterior papillary muscle.

The control AP 40-minute sample from 1 dog had sufficient extract to allow determination of ATP but not of ADP and AMP. The ATP results of this PP sample are included in the 40-minute mean. The total nucleotide values were obtained by summing the totals of the 11 animals on which data were available.

Mean differences between AP tissue and PP tissue were assessed after 15, 30, or 40 minutes of ischemia. Statistical comparisons were made with a two-tailed non-paired *t* test.

* $P = < 0.001$.

† $P = < 0.01$.

only a small fraction of the ATP lost. Thus, the depletion of the total adenine nucleotide pool presumably was due to degradation of the nucleotides to adenosine, inosine, or hypoxanthine.[12,34]

The adenylate charge ratio (ACR)* [35] of the control tissue was 0.90 but was reduced significantly to 0.76, 0.54, and 0.32 after 15, 30, and 40 minutes of ischemia, respectively (Table 2).

Cell Volume Regulation and High Energy Phosphate

Incubation of control slices caused no change in cell water, a 20% decrease in K^+ and Mg^{2+}, and a 46% increase in Na^{2+} (Table 3). Most but probably not all of the differences between control unincubated and incubated tissue can be attributed to changes within the cut cells on the edge of the slice.[25] However, part of the increase in slice Na^+ may be due to a slight increase in interstitial space and/or to potential surface contamination of the slice by media with a high Na^+ concentration.

After 60 minutes of incubation, the ATP of control slices was reduced from 5.57 to 2.80 μmole/g wet weight (Table 3, Text-figure 1). This decrease was associated with a decrease in the overall adenylate pool [36] and was most likely due to loss of adenine nucleotides during incubation, perhaps by washout of adenosine or inosine, without adequate resynthesis. Although the level of ATP was reduced at 1 hour, ATP synthesis was apparently sufficient for the metabolic demands of the incubated tissue since reserve high energy phosphate in the form of CP increased 221% by the end of 1 hour of incubation (Table 3, Text-figure 2).

The electrolyte and water contents of unincubated PP tissue following ischemia for 15, 30, 40, or 60 minutes were indistinguishable from those of the control unincubated AP shown in Table 3. Nevertheless, the capacity of the injured tissue to maintain volume during *in vitro* incubation was altered by all intervals of ischemia. Slices incubated after 15 minutes of ischemia *in vivo* showed a slight increase in water and Na^+ and a slight concomitant reduction in K^+ and Mg^{2+}. These changes became progressively more marked at 30, 40, and 60 minutes, so that at 60 minutes the concentrations of Na^+ and K^+ in the slices were approaching the concentrations in the media.

This progressive loss of cell volume regulation was associated with a progressively greater depletion of ATP and a progressive inability to

---

* $\frac{ATP + \frac{1}{2} ADP}{ATP + ADP + AMP} = ACR$

This ratio estimates the proportion of the total cellular adenine nucleotides which contain high energy phosphate. The ratio in normal cells is 0.85 to 0.9. If all the adenine nucleotide were ATP, the ratio would be 1.0; if it were all AMP, the ratio would be 0.

Table 3—Electrolytes, Water, and Metabolites of Control Myocardium and Incubated Slices of Control Myocardium and Myocardium Injured by Varying Periods of Ischemia

		Incubated tissue slices				
			PP			
			Minutes of Ischemia			
	Control tissue* from AP	AP	15	30	40	60
TTW (ml/100 g dry)	354.3 ± 4.56 (21)	345.5 ± 4.26 (20)	364.8 ± 5.17† (5)	372.0 ± 8.5† (6)	370.0 ± 11.6† (5)	425.5 ± 17.6§ (4)
IDS (ml/100 g dry)	—	83.7 ± 3.94 (20)	72.6 ± 3.8 (5)	88.3 ± 4.1 (6)	112.2 ± 14.4† (5)	189.5 ± 13.1§ (4)
Na (mmole/100 g)	18.5 ± 0.76 (20)	26.6 ± 0.94§ (19)	31.1 ± 1.29† (5)	38.7 ± 3.66§ (6)	46.1 ± 4.08§ (4)	57.0 ± 2.94§ (4)
K (mmole/100 g)	40.7 ± 0.59 (20)	29.4 ± 0.90§ (19)	27.0 ± 2.39 (5)	20.0 ± 2.16§ (6)	13.9 ± 4.84§ (4)	8.33 ± 1.09§ (4)
Mg (mmole/100 g)	5.1 ± 0.10 (21)	4.2 ± 0.14§ (19)	4.1 ± 0.18 (5)	3.3 ± 0.29‡ (6)	3.0 ± 0.38‡ (4)	2.6 ± 0.27§ (4)
ATP (μmole/g wet)	5.57 ± 0.19 (21)	2.80 ± 0.16§ (21)	1.12 ± 0.15§ (5)	0.90 ± 0.29§ (6)	0.71 ± 0.26§ (5)	0.11 ± 0.03§ (4)
CP (μmole/g wet)	2.10 ± 0.13 (21)	6.74 ± 0.41§ (21)	4.49 ± 0.92† (5)	3.34 ± 1.08‡ (6)	2.53 ± 0.99§ (5)	0.33 ± 0.13§ (4)

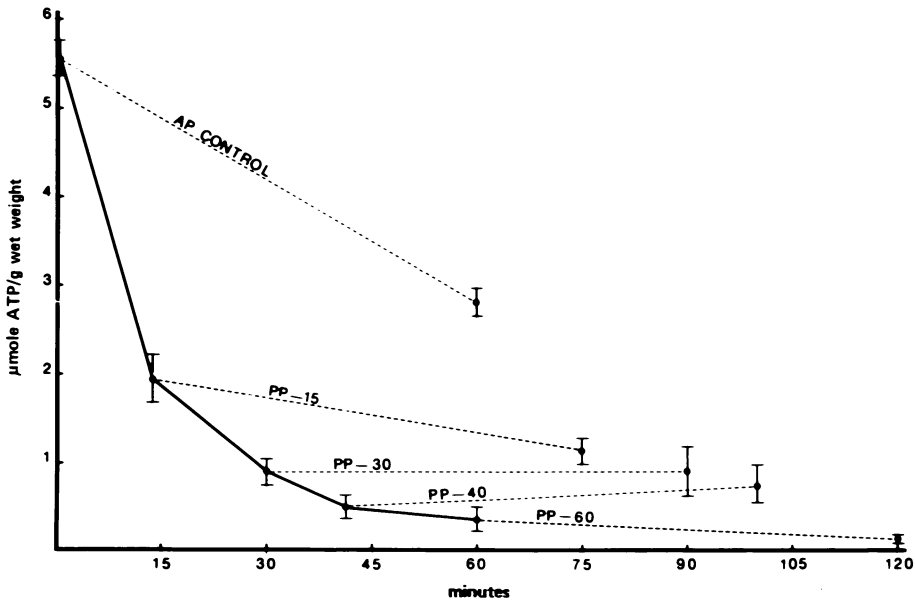
The dogs included in this table are those in which high energy phosphate was determined. Except for IDS, which was assessed by a two-tailed paired *t* test, statistical comparisons were made with a two-tailed non-paired *t* test. Mean differences were assessed between AP slices before and after 60 minutes of incubation and between incubated PP and incubated AP slices.

* The ATP and CP of unincubated PP tissue at 15, 30, 40, and 60 minutes are given in Table 1. The water and electrolyte content of PP prior to incubation is not shown because it was virtually identical to unincubated control (AP) tissue.

† $P = < 0.05$.

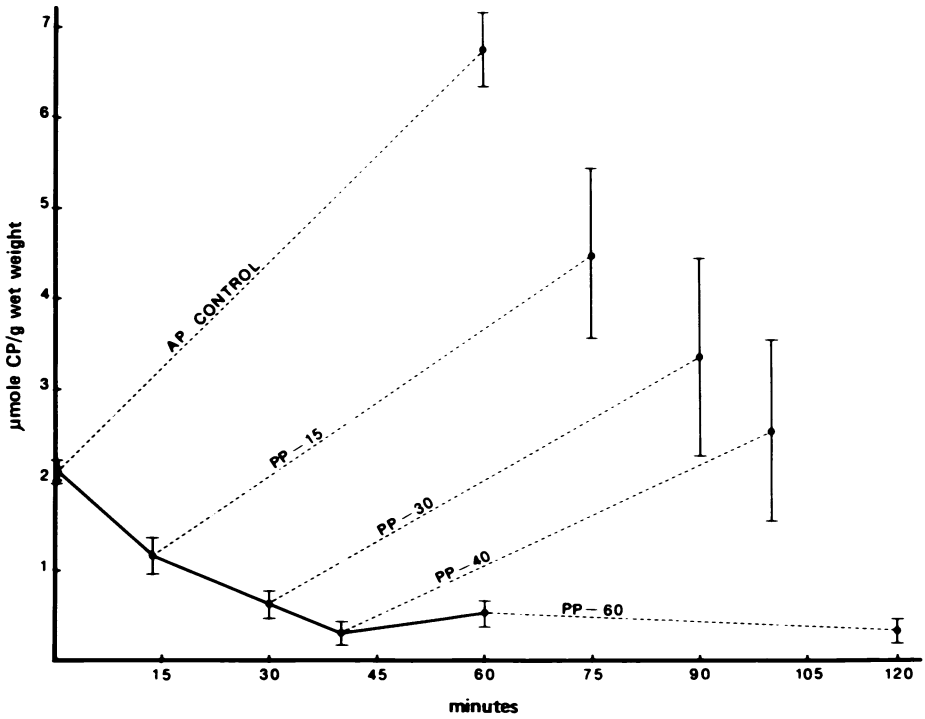
‡ $P = < 0.01$.

§ $P = < 0.001$.



TEXT-FIGURE 1—Effect of 60-minute incubation in oxygenated KRP on ATP of posterior papillary (PP) muscle slices after 15, 30, 40, and 60 minutes of *in vivo* ischemia. The ATP content of nonischemic PP and anterior papillary muscle (AP) from control dogs are identical. The *solid line* shows the ATP content of the tissue slices prior to incubation. (See Table 1.) Uninjured slices of the AP lost 2.44 µmole ATP during incubation (*dashed lines*). Some of this loss is from cut cells on the edge of the slice. The adenylate charge was approximately 0.8 at the end of the incubation. Injured slices started with markedly reduced ATP contents and were unable to resynthesize it during incubation.

resynthesize CP (Text-figures 1 and 2). The solid lines depict the ATP and CP of the PP at the time incubation was begun and illustrate the change in high energy phosphate found *in vivo* after 15, 30, 40, and 60 minutes of ischemia. The dotted lines indicate the capacity of the slices of injured PP to maintain ATP (Text-figure 1) or to synthesize CP (Text-figure 2) while incubating *in vitro*. In all injured slices, as in the control slices, no restoration of ATP content was observed. On the other hand, slices injured by 15 minutes of ischemia synthesized 3.31 µmole CP. Conversely, after 60 minutes of ischemia, incubated slices had virtually no ATP and could not synthesize CP. At 40 minutes, net CP synthesis occurred in only 2 of 5 animals. These showed slice CP levels of 4.17 and 5.48 µmole/g; the other three animals showed slice CP levels of 0.5, 0.7 and 1.8 µmole/g, respectively. The latter three dogs also showed the most severe decline in slice volume control.



TEXT-FIGURE 2—Capacity of PP slices to synthesize CP after 15, 30, 40, and 60 minutes of *in vivo* ischemia. Nonischemic AP slices had low CP before incubation because of the sampling technique but resynthesized almost 5.0 µmole CP/g wet weight during incubation in oxygenated KRP (*dashed line*). As the period of *in vivo* ischemia was extended, the CP level in the PP decreased (*solid line*). Incubation after 15, 30, and 40 minutes of *in vivo* ischemia resulted in some CP resynthesis, but no synthesis was noted after 60 minutes of *in vivo* ischemia. The large standard errors after 30 and 40 minutes of ischemia reflect the fact that 2 of 6 animals after 30 minutes and 3 of 5 animals after 40 minutes of ischemia showed virtually no CP synthesis.

Discussion

These experiments show that total adenine nucleotides decrease quickly and markedly in severely ischemic myocardium (Text-figure 1). By 15 minutes, the tissue ATP is reduced by 65%; by 40 minutes, less than 10% remains. This loss of ATP is accompanied by a moderate decrease in ADP and a 600% rise in AMP. However, the increase in tissue AMP accounts for only a small fraction of the nucleotide lost. Although rapid loss of ATP during ischemic or anoxic injury has been observed previously,[1-3,5-8] in general, the losses have not been as marked as those noted in this study. The greater ATP depletion noted in the present study is probably due to our technique of identifying severely ischemic tissue to avoid contamination with normal or mildly ischemic tissue.

Evaluation of the Sampling Technique

In the present study, the method of sampling was designed to allow identification of severely ischemic myocardium. This objective was achieved by injecting the fluorescent dye thioflavin S intravenously just prior to excision of the heart. When viewed under ultraviolet light, nonischemic or mildly ischemic myocardium, perfused with the dye, is brightly fluorescent. Severely ischemic areas are nonfluorescent and thereby readily identified. The primary advantage of this technique is that it makes it possible to sample tissue known to be severely ischemic. Contamination with tissue receiving significant collateral flow is avoided.

The major limitation of this technique is that some anaerobic metabolism occurs during the 2 to 3 minutes of time required to cool the excised heart. In effect, "nonischemic" myocardium has been ischemic for a brief period and metabolite levels are altered accordingly. The extent to which this occurred can be evaluated by comparison of the control metabolites, obtained by this technique, with metabolite levels in quick-frozen myocardium.[1,7,8] CP is depleted to 25 to 30% and both lactate and G6P increase significantly during the period of cooling. On the other hand, ATP is relatively well maintained, presumably because of resynthesis from CP via creatine kinase.

However, the purpose of the present study was not to compare metabolite levels in injured muscle with normal levels but rather to evaluate the relationship of metabolite levels in control or ischemic myocardium to the capacity of the cells to maintain cell structure and cell volume regulation *in vitro*. Viable slices cannot be cut from quick-frozen myocardium, and a brief period of anaerobic metabolism during cooling is a necessary prerequisite for slice preparation.

Since metabolite changes occur most rapidly during the first few minutes of ischemia, the slow sampling technique should have had only a minimal effect on metabolite levels within the ischemic acontractile myocardium. In effect, for example, the metabolite levels reached after 40 minutes of ischemia plus cooling might be equivalent to metabolite levels after 41 to 42 minutes of ischemia followed by quick freezing for sampling—an insignificant difference.

Review of the Posterior Papillary Muscle Model of Ischemic Injury

Occlusion of the circumflex coronary artery in the dog results in severe ischemia of the posterior papillary muscle [37]; collateral flow to this muscle is usually less than 10% of control arterial flow.[38] The resulting oxygen deficiency quickly causes a shift in myocardial metabolism to anaerobic

glycolysis,[37] which becomes the chief source of new high energy phosphate.

The transition to anaerobic glycolysis is associated with a prompt and marked decrease in CP, much of which is converted to ATP via the action of creatine kinase. Within 60 seconds, only 12% of the initial CP remains within the ischemic tissue.[1] By this time, the myocardial cells have ceased contracting [39] and electrocardiographic changes are well developed.[40] Although the cessation of contraction reduces one of the major drains on tissue ATP, the ATP content continues to decrease.[1] Presumably, ATP is used to maintain cell volume, ionic gradients, the excitable state,[10] and a variety of other ATP-dependent reactions which can utilize ATP under anaerobic conditions (Text-figure 3).

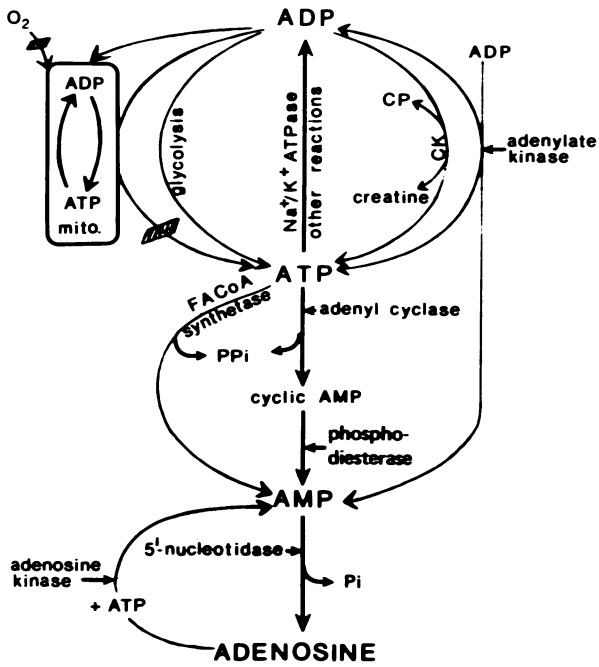
AMP is the end product for many of these reactions and is important because it is further degraded by 5′-nucleotidase, an enzyme located in the sarcolemma of the myocyte. This enzyme dephosphorylates AMP to form adenosine, which can diffuse to the extracellular fluid. Adenosine and further catabolic products of adenine nucleotide metabolism such as inosine and hypoxanthine appear in the venous effluent from ischemic muscle within the first few seconds of the onset of ischemia [12]; this probably is the principal route by which adenine nucleotides are lost from the cell (Text-figure 3).

Despite the rapid depletion of adenine nucleotides, myocardial cells will survive 15 minutes of ischemia.[41-44] Reperfusion at this time is followed by resumption of contraction, the disappearance of the electrocardiographic changes of ischemia, and absence of necrosis by histologic evaluation 1 to 4 days later.[44]

Relation Between Adenine Nucleotide Depletion and Cell Death

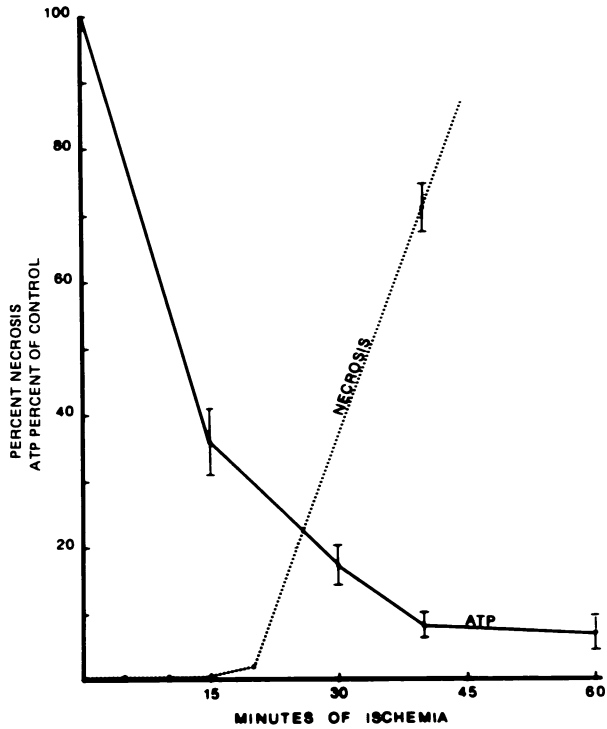
Irreversible injury first develops in the PP muscle after 20 minutes of ischemia; by 40 to 60 minutes of ischemia, most of the cells of the PP muscle and surrounding subendocardial myocardium are irreversibly injured. Reperfusion at these times is followed by the prompt development within 2 minutes of explosive cell swelling, contraction bands, and calcification of mitochondria.[42,43] Study of the PP by light microscopy after several days of reperfusion confirms the irreversibility of the injury in that the tissue now contains a mixture of necrotic cells and granulation tissue.[44,45] At 20 minutes only 2% of the PP muscle cells became necrotic,[46] but after 40 minutes of ischemia 72% and by 60 minutes virtually 100% of the cells of the PP muscle die injured.[47] Thus, irreversible injury in the PP develops rapidly between 20 and 40 minutes (Text-figure 4).

Cell death first occurs when ATP levels are at approximately 20% of



TEXT-FIGURE 3—Diagram of nucleotide metabolism in severely ischemic myocardium. The reactions which take place in the markedly hypoxic environment of the zone of severe ischemia are emphasized. In this zone, mitochondrial respiration is assumed to be inoperative because of the virtual absence of oxygen. After contraction ceases, the reactions utilizing ATP to produce ADP include the Na/K ATPase of the cell membrane and a variety of ATPases and protein kinases of the cell interior. The principal means of generating ATP from ADP after CP supplies for the action of creatine kinase (CK) are exhausted is anaerobic glycolysis or adenylate kinase (myokinase). The routes by which AMP is generated are emphasized because of the crucial role of 5'-nucleotidase in the loss of adenine nucleotides from ischemic cells. This enzyme of the sarcolemma dephosphorylates adenosine, which then diffuses to the extracellular space. It seems unlikely that enough ATP is available in areas of severe ischemia to support the action of adenosine kinase to convert adenosine to AMP and then to ATP via adenylate kinase. The principal reactions producing AMP include fatty acid acyl-CoA synthetase (FA Co-A synthetase), adenylate kinase, and the formation and degradation of cyclic AMP via the action of adenyl cyclase and phosphodiesterase. Approximately 50% of the adenine nucleotides of a myocyte are in the mitochondria, and the means by which these are degraded is unknown. Since the cellular ATP drops quickly in ischemia, they presumably reach the sarcoplasm and participate in the reactions shown.

control (Text-figure 4). By the time the tissue ATP is less than 10% of the control value, most of the cells of the PP are dead. Accordingly, ATP and adenine nucleotide depletion correlate closely with the development of irreversible injury in ischemically injured myocardium. The time course of the decreasing adenylate charge ratio also correlates with the timing of cell death since this ratio was 0.74 in the reversible phase and 0.32 when most of the cells are irreversibly injured. An ACR of 0.5 has been considered to be critical for cell viability.[35,48]



TEXT-FIGURE 4—The relationship between the level of ATP remaining in the PP and the amount of irreversible injury in this muscle after various periods of ischemia is plotted in this graph. The ATP data are from Table 1. The percent necrosis data are from a series of experiments in which the amount of necrosis found after 5 to 60 minutes of temporary ischemia and 2 to 4 days of arterial reperfusion was quantitated by histologic techniques.[46-47] Intervals of ischemia of 15 minutes or less result in no irreversible injury. After 20 minutes of ischemia, only 2%, on the average, of the portion of the PP used to obtain the ATP curve is necrotic.[46] After 40 minutes of ischemia, the upper half of the projecting PP shows 72±3% necrosis.[47] The 20- and 40-minute points are based on 15 and 24 dogs, respectively. Examination of the ATP necrosis curves shows that myocardial cells tolerate ATP levels of 38% of control without developing necrosis. The level of ATP at which necrosis first appeared was approximately 20%, and depletion to <10% of control ATP was associated with extensive necrosis within the PP tissue.

Although the results of these experiments suggest that, in myocardium, a depressed level of total adenine nucleotides, especially ATP, is associated with the appearance of irreversible injury, persistently low cellular ATP is not lethal in some tissues. For example, hepatocytes of rats intoxicated with ethionine survive for long periods with 20% of control ATP.[49] However, there are important differences between ischemic injury in heart and ethionine intoxication in liver. First the liver cells maintain an equilibrium level of 20% ATP by continuous synthesis of ATP to replace that lost due to the slow turnover of S-adenosylethionine. Moreover, even when hepatic ATP is 20% of control, it seems likely that there

are sufficient ATP molecules present to reach the Km of most ATP-dependent reactions.[50] The ischemic heart, on the other hand, cannot synthesize nucleotides either *de novo* or by salvage pathways because of anaerobiasis and the lack of arterial flow to provide precursors.

Studies of ischemic injury in certain other well-differentiated tissues have failed to demonstrate a close association between depressed adenine nucleotide levels and the onset of irreversible injury.[51-54] Brain, kidney, and liver all can show striking degrees of ATP depletion with minimal irreversible injury. However, when these tissues are reperfused with arterial blood at a time when the ATP is very low, they can resynthesize a significant amount of ATP via salvage pathways as well as via *de novo* synthesis.[52,53] These studies suggest that the capacity to resynthesize ATP and adenine nucleotides after reperfusion of the tissue is a better measure of cell viability than is the level to which these nucleotides are depleted per se.

On the other hand, *in vitro* studies of cell injury utilizing tissues such as Ehrlich ascites tumor cells, toad bladder, or flounder kidney slices show a close relationship between low ATP and cell death as manifest by disintegration of the cells.[55-58] This also is true in cultures of embryonic mouse heart.[59,60] However, if lack of adenine nucleotides is the cause of irreversibility in these isolated systems, the mechanism by which cell death occurs remains unknown.

It is of interest that depression of uridine triphosphate (UTP) levels to 20% for a few hours by *d*-galactosamine induces death of aerobic hepatocytes by interfering with macromolecular synthesis of cell membrane components.[50,61] These experiments have shown that the cell membrane lesion is reversible for 60 to 120 minutes if uridine is supplied to the hepatic cells for salvage synthesis of UTP. Thus, the number of molecules of nucleotide available and the duration of the period of depressed levels of nucleotide both are important features of cell injury in this model. The relationship of uridine and guanosine nucleotides to irreversibility has not been studied in myocardial ischemic injury.

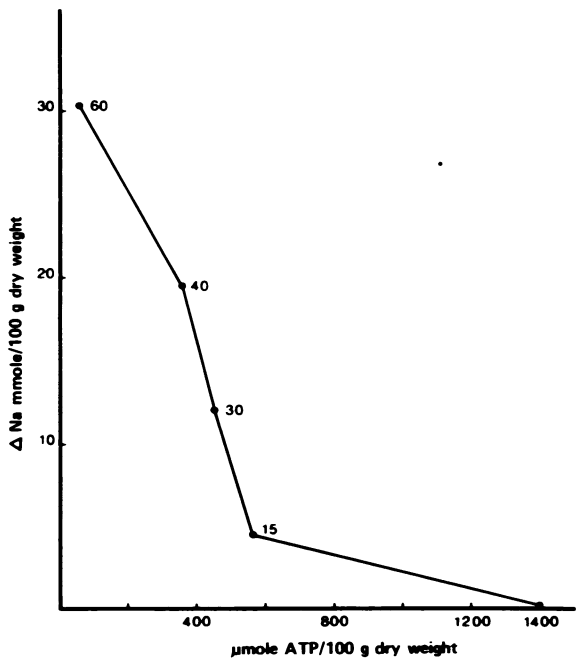
Results of recent work of Gazitt et al [62,63] suggest one potential molecular mechanism through which ATP depletion could lead to the disruption of the plasma membrane of ischemic cells. Studies of intact ATP-depleted erythrocytes have shown that the membrane of such cells is much more susceptible to the action of phospholipase and sphingomyelinase than is the membrane of normal cells. Their data indicate that defective phosphorylation of membrane proteins by ATP is the cause of the increased susceptibility. Thus, absence of ATP for phosphorylation of membrane proteins indirectly may lead to disruption of the sarcolemma.

Cell Volume Control and Electrolytes in Ischemia

The loss of ATP and/or the inability of the injured cells to resynthesize ATP could explain the associated defects in volume control observed in injured slices incubated at 37 C in oxygenated KRP (Text-figure 5). The Na^+/K^+ ATPase of the sarcolemma appears to be essential for maintaining cell volume since myocardial cells lose K^+, gain Na^+, and swell markedly when incubated with 10^{-3} M ouabain,[64] a specific inhibitor of this enzyme. This ATPase has been most thoroughly studied in red cells and nerve.[65] It is phosphorylated in the cell membrane during the course of transport of Na^+ and K^+ across the sarcolemma. This phosphorylation requires intracellular ATP, and Na^+/K^+ exchange does not occur without this step.[65] It follows that ATP depletion near the sarcolemma would prevent cell volume control. However, the level of intracellular ATP required for the function of Na^+/K^+ ATPase in a myocardial cell is unknown.

Synthesis of CP by Slices as a Measure of Irreversibility

The data in Text-figure 2 show that cells reversibly injured by 15 minutes of ischemia could resynthesize significant amounts of CP. Similar results have been noted in studies of ischemic injury in perfused hearts.[66] Such hearts, when made anoxic, lose adenine nucleotides and CP but,



TEXT-FIGURE 5—The increase over control in Na^+ content of tissue slices incubated for 60 minutes is plotted vs the mean ATP content of the slices. The period of *in vivo* ischemia in minutes is indicated by 15, 30, 40, and 60. Note that reversibly injured cells (15 minutes) excluded Na^+ efficiently compared with slices of tissue injured by 30 and 40 minutes of *in vivo* ischemia. There was only a small change in final ATP content between slices which excluded vs those which could not exclude Na^+ efficiently. The ATP is reported on the basis of dry weight because of the edema which developed during incubation of the injured tissue (Table 3).

when reoxygenated after brief periods of anoxia, show net CP synthesis. This suggests that the ATP/ADP ratio has been sufficiently restored to phosphorylate creatine via creatine kinase although the equilibrium of this reaction at physiologic pH is far on the side of ATP formation. In contrast, cells irreversibly injured by 60 minutes of ischemia in the present study could not resynthesize CP. This failure of CP synthesis could be due to the very low intracellular ATP, a low adenylate charge ratio, absence of creatine, inactivation or inhibition of the action of creatine kinase, inadequate intracellular supplies of the cofactor Mg^{2+}, or other unknown factors. Further work will be required to establish the mechanism.

Clinical Implications

If the critical role of the adenine nucleotide pool in the maintenance of cell viability suggested by this study is confirmed, then any maneuver which delays the loss of ATP and adenine nucleotides from ischemic myocardial cells could delay the onset of lethal cell injury. The beneficial effects of hypothermia [60] and verapamil [21,67] on myocardial tissue or intact hearts might be explained by this mechanism. It seems likely that development of methods to inhibit the action of 5′-nucleotidase or the principal reactions leading to the formation of AMP also could delay the appearance of cell death in severe ischemia. The critical role of this enzyme in the depletion of adenine nucleotides from ischemic cells is summarized in Text-figure 3.

Study of the development of the irreversible state in well-differentiated mammalian cells is complex. Many interrelated events occur simultaneously, resulting in failure of function or lethal injury. Thus, proof that the temporal relationship between high energy phosphate depletion and cell death is causal rather than simply an associated event will require identification of the reaction or reactions, the absence of which causes the injury to become irreversible.

Additional Note

Allison et al [68] reported results of an experiment in which high energy phosphate was measured in quick-frozen needle biopsy samples of subendocardial myocardial tissue from the center of the area of ischemia in the same occlusion model used in our experiments. The findings were generally similar to those reported in Tables 1 and 2 of this paper, except that in their study, ATP and Σ AD depletion from subendocardial tissue occurred more slowly in that it took 3 minutes for CP and 18.8 minutes for ATP to reach 50% of the initial concentration. This delay presumably was related to the method of sampling employed, but the general trend was identical to that reported in our experiments.

References

1. Braasch W, Gudbjarnason S, Puri PS, Ravens KG, Bing RJ: Early changes in energy metabolism in the myocardium following acute coronary artery occlusion in anesthetized dogs. Circ Res 23:429–438, 1968
2. Williamson JR: Glycolytic control mechanisms. II. Kinetics of intermediate changes during the aerobic-anoxic transition in perfused rat heart. J Biol Chem 241:5026–5036, 1966
3. Kübler W, Spieckermann PG: Regulation of glycolysis on the ischemic and the anoxic myocardium. J Mol Cell Cardiol 1:351–377, 1970
4. Wollenberger A, Krause E-G: Metabolic control characteristics of the acutely ischemic myocardium. Am J Cardiol 22:349–359, 1968
5. Neely JR, Rovetto MJ, Whitmer JT, Morgan HE: Effects of ischemia on function and metabolism of the isolated working rat heart. Am J Physiol 225:651–658, 1973
6. Dunn RB, Griggs DM Jr: Transmural gradients in ventricular tissue metabolites produced by stopping coronary blood flow in the dog. Circ Res 37:438–445, 1975
7. Gudbjarnason S, Mathes P, Raven KG: Functional compartmentation of ATP and creatine phosphate in heart muscle. J Mol Cell Cardiol 1:325–339, 1970
8. Jones CE, Thomas JX, Parker JC, Parker RE: Acute changes in high energy phosphates, nucleotide derivatives and contractile force in ischaemic and non-ischaemic canine myocardium following cornoary occlusion. Cardiovasc Res 10:275–282, 1976
9. Sayen JJ, Sheldon WF, Pierce G, Kuo P: Polarographic oxygen, the epicardial electrocardiogram and muscle contraction in experimental acute regional ischemia of the left ventricle. Circ Res 6:779–798, 1958
10. Langer GA: Ionic movements and the control of contraction. The Mammalian Myocardium. Edited by GA Langer. AJ Brady. New York, John Wiley & Sons, Inc., 1974, pp 193–217
11. Dobson JG Jr, Mayer SE: Mechanisms of activation of cardiac glycogen phosphorylase in ischemia and anoxia. Circ Res 33:412–420, 1973
12. Berne RM, Rubio R: Adenine nucleotide metabolism in the heart. Circ Res 35 (Suppl 3):109–120, 1974
13. Wood JM, Hutchings AE, Brachfeld N: Lipid metabolism in myocardial cell-free homogenates. J Mol Cell Cardiol 4:97–111, 1972
14. Larner J: Mechanisms of regulation of glycogen synthesis and degradation. Circ Res 38 & 39 (Suppl 1):2–7, 1978
15. Scheuer J: The effect of hypoxia on glycolytic ATP production. J Mol Cell Cardiol 4:689–692, 1972
16. Scheuer J, Stezoski SW: Protective role of increased myocardial glycogen stores in cardiac anoxia in the rat. Circ Res 27:835–849, 1970
17. Pool PE, Covell JW, Chidsey CA, Braunwald E: Myocardial high energy phosphate stores in acutely induced hypoxic heart failure. Circ Res 19:221–229, 1966
18. Neely JR, Morgan HE: Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. Annu Rev Physiol 36:413–459, 1974
19. Rovetto MJ, Whitmer JT, Neely JT: Comparison of the effects of anoxia and whole heart ischemia on carbohydrate utilization in isolated working rat hearts. Circ Res 32:699–711, 1973
20. Williamson JR, Schaffer SW, Ford C, Safer B: Contribution of tissue acidosis to ischemic injury in the perfused rat heart. Circulation 53(Suppl 1):3–14, 1976
21. Nayler WG, Grau A, Slade A: A protective effect of verapamil on hypoxic heart muscle. Cardiovasc Res 10:650–662, 1976
22. Hearse DJ, Stewart DA, Chain EB: Diabetes and the survival and recovery of the anoxic myocardium. J Mol Cell Cardiol 7:397–415, 1975

23. Kloner RA, Ganote CE, Reimer KA, Jennings RB: Distribution of coronary arterial flow in acute myocardial ischemia. Arch Pathol 99:86–94, 1975
24. Kloner RA, Reimer KA, Jennings RB: Distribution of coronary collateral flow in acute myocardial ischemic injury: Effect of propranolol. Cardiovas Res 10:81–90, 1976
25. Grochowski EC, Ganote CE, Hill ML, Jennings RB: Experimental myocardial ischemic injury. I. A comparison of Stadie-Riggs and free-hand slicing techniques on tissue ultrastructure, water and electrolytes during *in vitro* incubation. J Mol Cell Cardiol 8:173–187, 1976
26. Ganote CE, Jennings RB, Hill, ML, Grochowski EC: Experimental myocardial ischemic injury. II. Effect of *in vivo* ischemia on dog heart slice function *in vitro*. J Mol Cell Cardiol 8:189–204, 1976
27. Lowry OH, Passonneau JV: A Flexible System of Enzymatic Analysis. New York, Academic Press, Inc., 1972, pp 94–201
28. Lamprecht W, Trautschold I: Determination of ATP with hexokinase and glucose-6-phosphate dehydrogenase. Methods of Enzymatic Analysis. Edited by HV Bergmeyer. New York, Academic Press, Inc., 1974, pp 2101–2110
29. Jawouk D, Gruber W, Bergmeyer HV: Adenosine-5-diphosphate and adenosine-5'-monophosphate.[28] pp 2127–2131
30. Jennings RB, Crout JR, Smetters GW: Studies on distribution and localization of potassium in early myocardial ischemic injury. Arch Pathol 63:586–592, 1957
31. Jennings RB, Moore CB, Shen AC, Herdson PB: Electrolytes of damaged myocardial mitochondria. Proc Soc Exp Biol Med 135:515–522, 1970
32. Luft JH: Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414, 1961
33. Jennings RB, Ganote CE: Structural changes in myocardium during acute ischemia. Circ Res 35 (Suppl 3):156–172, 1974
34. Thomas RA, Rubio R, Berne RM: Comparison of the adenine nucleotide metabolism of dog atrial and ventricular myocardium. J Mol Cell Cardiol 7:115–123, 1975
35. Atkinson DE: The energy charge of the adenylate pool as a regulatory parameter: Interaction with feedback modifiers. Biochemistry 7:4030–4034, 1968
36. Jennings RB, Hill ML, Klotman S: Unreported data
37. Jennings RB, Ganote CE, Reimer KA: Ischemic tissue injury. Am J Pathol 81:179–198, 1975
38. Reimer KA, Lowe JE, Jennings RB: The wavefront phenomenon of ischemic cell death. Acta Med Scand (In press)
39. Lamprecht W: The effect of acute and chronic ischemia on myocardial contractility. The Etiology of Myocardial Infarction. Edited by TN James, JW Keyes. Boston, Little, Brown & Co., 1963, pp 393–410
40. Jennings RB, Wartman WB: Production of an area of homogeneous myocardial infarction in the dog. AMA Arch Pathol 63:580–585, 1957
41. Jennings RB, Sommers HM, Herdson PB, Kaltenbach JP: Ischemic injury of myocardium. Ann NY Acad Sci 156:61–78, 1969
42. Whalen DA Jr, Hamilton DG, Ganote CE, Jennings RB: Effect of a transient period of ischemia on myocardial cells. I. Effects on cell volume regulation. Am J Pathol 74:381–398, 1974
43. Kloner RA, Ganote CE, Whalen DA Jr, Jennings RB: Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. Am J Pathol 74:399–422, 1974
44. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H: Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Arch Pathol 70:68–78, 1960

45. Reimer KA, Rasmussen MM, Jennings RB: Reduction by propranolol of myocardial necrosis following temporary coronary artery occlusion in dogs. Circ Res 33:353–363, 1973
46. Sommers HM, Jennings RB: Ventricular fibrillation and myocardial necrosis after transient ischemia: Effect of treatment with oxygen, procainamide, reserpine, and propranolol. Arch Intern Med 129:780–789, 1972
47. Reimer KA, Jennings RB: Unreported data
48. Ridge JW: Hypoxia and the energy change of the cerebral adenylate pool. Biochem J 127:351–355, 1972
49. Farber E: ATP and cell integrity. Fed Proc 32:1534–1539, 1973
50. Decker K: Quantitative aspects of biochemical mechanisms leading to cell death. Pathogenesis and Mechanisms of Liver Cell Necrosis. Edited by D Klepper. Baltimore, University Park Press, 1975, pp 45–56
51. Vogt MT, Farber E: On the molecular pathology of ischemic renal cell death: Reversible and irreversible cellular and mitochondrial metabolic alterations. Am J Pathol 53:1–26, 1968
52. Kleihues P, Kobayashi K, Hossmann KA: Purine nucleotide metabolism in the cat brain after one hour of complete ischemia. J Neurochem 23:417–425, 1974
53. Kleihues P, Hossmann KA, Pegg AE, Kobayashi K, Zimmermann V: Resuscitation of the monkey brain after one hour complete ischemia. III. Indications of metabolic recovery. Brain Res 95:61–73, 1975
54. Busch EW, Habel G, Wichert P von: Restitution von Adenosinphosphaten im Lebergewebe während der Wiederdurchblutung nach ischämischen Belastungen bis zu 5 std. Dauer Biochem Z 341:85–96, 1964
55. Ginn FL, Shelburne JD, Trump BF: Disorders of cell volume regulation. I. Effects of inhibition of plasma membrane adenosine triphosphatase with ouabain. Am J Pathol 53:1041–1071, 1968
56. Laiho KU, Trump BF: Relationship of ionic, water, and cell volume changes in cellular injury of ehrlich ascites tumor cells. Lab Invest 31:207–215, 1974
57. Laiho KU, Trump BF: Studies on the pathogenesis of cell injury. Effects of inhibitors of metabolism and membrane function on the mitochondria of ehrlich ascites tumor cells. Lab Invest 32:163–182, 1975
58. Trump BF, Mergner WJ, Kahng MW, Saladino AJ: Studies on the subcellular pathophysiology of ischemia. Circulation 53(Suppl 1):17–26, 1976
59. Ingwall JS, DeLuca M, Sybers HD, Wildenthal K: Fetal mouse hearts: A model for studying ischemia. Proc Natl Acad Sci USA 72:2809–2813, 1975
60. Roeske WR, Ingwall JS, DeLuca M, Sybers HD: Thermal interventions in the ischemic fetal mouse heart (FMH). Circulaltion 51 & 52 (Suppl 2):2–23, 1975
61. Farber JL, El-Mofty SK: The biochemical pathology of liver cell necrosis. Am J Pathol 81:237–250, 1975
62. Gazitt Y, Loyter A, Reichler Y, Ohad I: Correlation between changes in the membrane organization and susceptibility to phospholipase C attack by ATP depletion of rat erythrocytes. Biochim Biophys Acta 419:479–492, 1976
63. Gazitt Y, Ohad I, Loyter A: Phosphorylation and dephosphorylation of membrane proteins as a possible mechanism for structural rearrangement of membrane components. Biochim Biophys Acta 436:1–14, 1976
64. Jennings RB: Unreported data
65. Hoffman JF: Ionic transport across the plasma membrane. Cell Membranes: Biochemistry, Cell Biology and Pathology. Edited by G Weissmann, R Claiborne. New York, Hospital Practice, 1975, pp 95–103
66. Reibel DK, Rovetto M: Myocardial adenine nucleotide utilization and mechanical function following anoxia and ischemia in the isolated perfused rat heart. Fed Proc 36:582, 1977

67. Reimer KA, Lowe JE, Jennings RB: Effect of the calcium antagonist verapamil on necrosis following temporary coronary artery occlusion in dogs. Circulation 55:581–587, 1977
68. Allison TB, Ramey CA, Holsinger JW, Jr: Transmural gradients of left ventricular tissue metabolites after circumflex artery ligation in dogs. J Mol Cell Cardiol 9:837–852, 1977

Acknowledgments

Surgical assistance was provided by Nancy J. Kramer and preparation of thin sections for electron microscopy was done by Henry Estrada and Michael Hale.

[Illustrations follow]

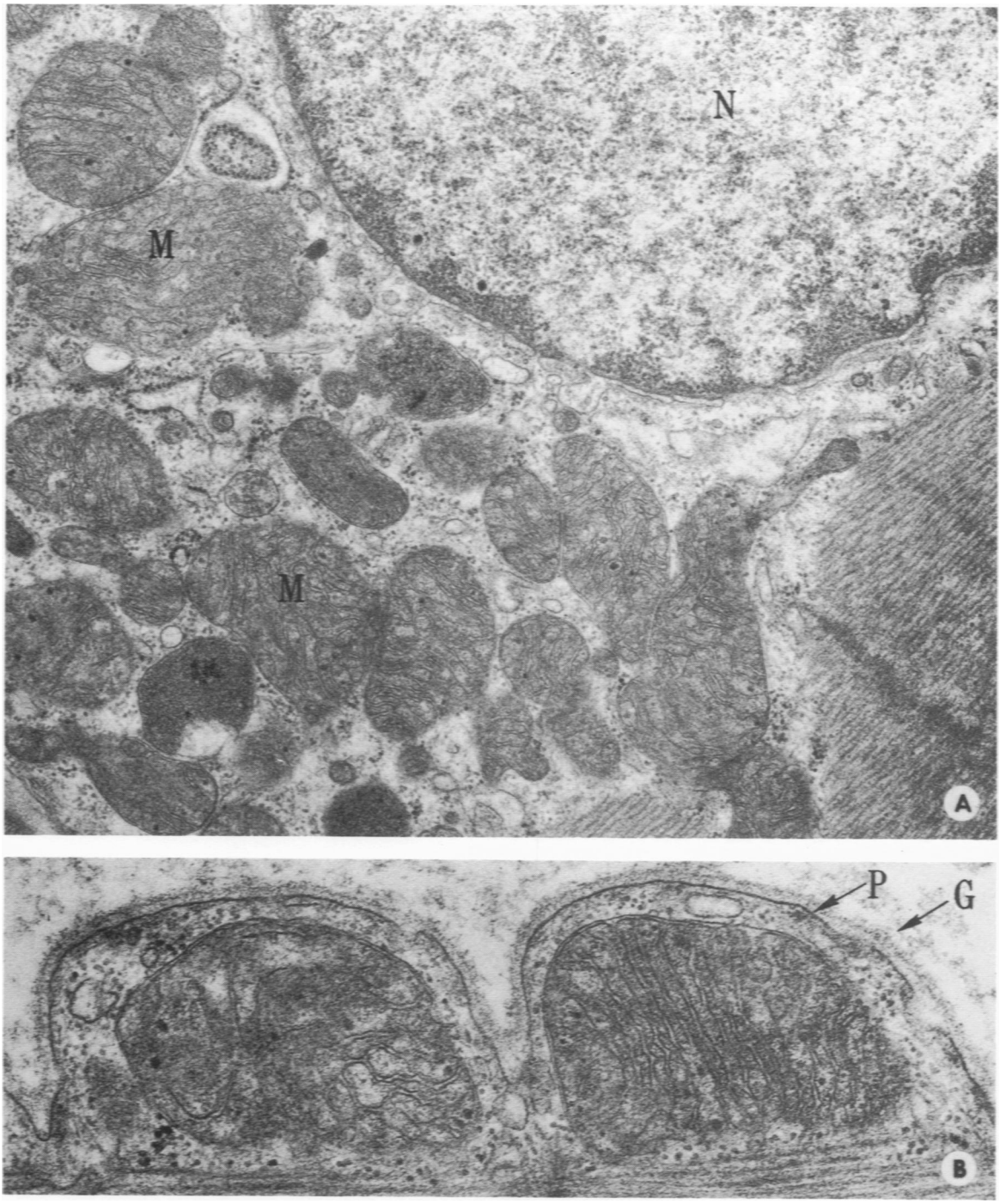
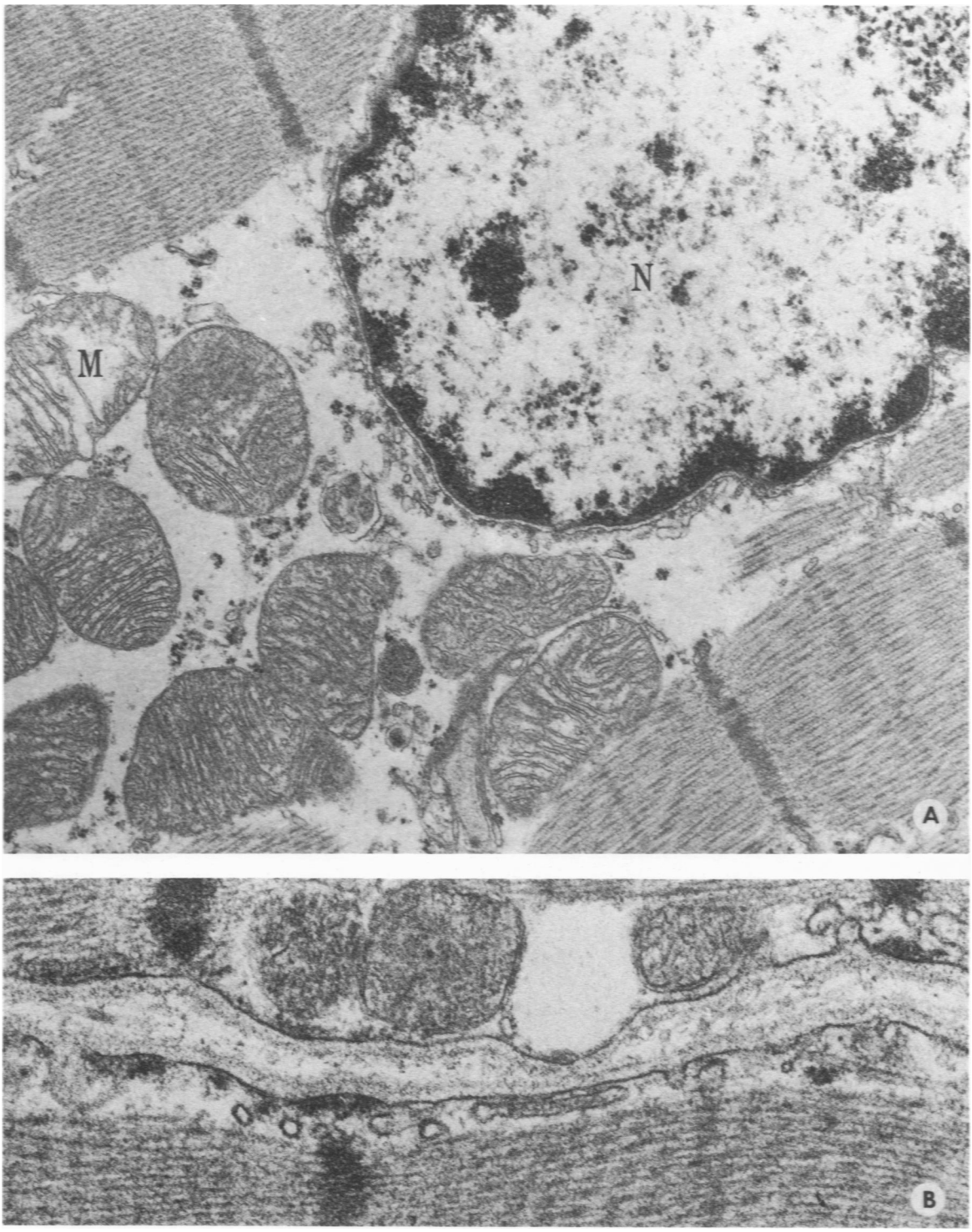


Figure 1—Nonischemic anterior papillary muscle. **A**—The nuclear chromatin (*N*) is evenly arranged. The mitochondria (*M*) are dense and contain occasional matrix granules. Glycogen is present but faintly stained as usual in tissue fixed in glutaraldehyde. **B**—The sarcolemma is scalloped over two contracted myofibrils. The plasma membrane (*P*) and glycocalyx (*G*) are typical of control myocardium. (**A**, × 31,000; **B**, × 50,000) (with printing reduction of 13%)

Figure 2—Posterior papillary muscle after 15 minutes of ischemia. **A**—The significant alterations from control include margination of nuclear chromatin (*N*) and slight mitochondrial swelling. One markedly swollen mitochondrion (*M*) is present. There appears to be less glycogen than in control myocardium. **B**—The sarcolemma is flattened over relaxed myofibrils. However, the plasma membrane and glycocalyx appear intact. (**A**, × 31,000; **B**, × 74,000) (with printing reduction of 13%)


N
M
A
B

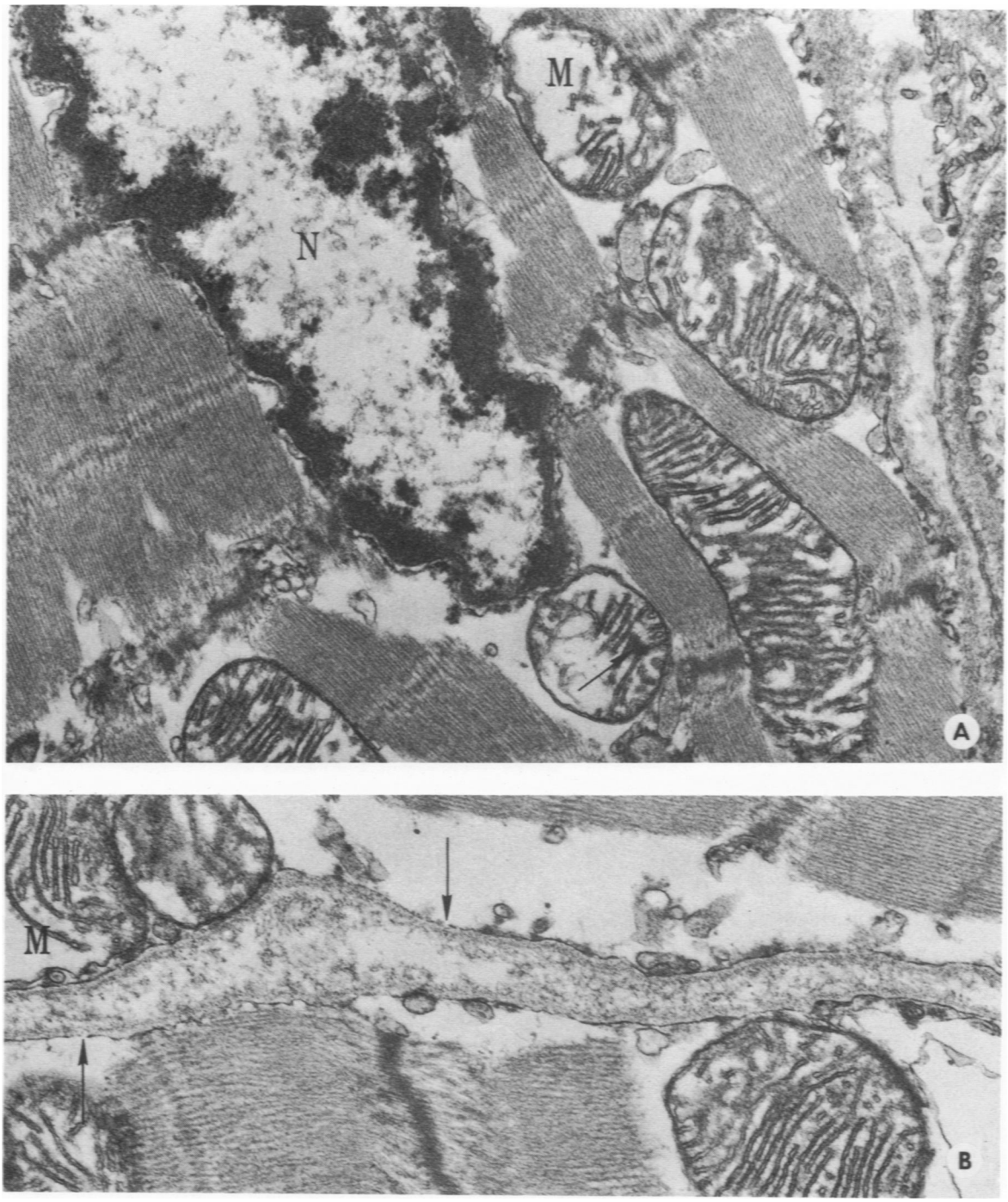


Figure 3—Posterior papillary muscle after 40 minutes of ischemia. **A**—The significant alterations from control include the marked margination of nuclear chromatin (*N*) and mitochondrial swelling (*M*) with the appearance of a clear matrix which often contains amorphous matrix densities (*arrow*). No glycogen is detectable. **B**—Several small breaks are visible in the plasma membrane although the glycocalyx appears continuous. Swollen mitochondria are present (*M*). The myofibrils are relaxed and show I bands. (**A**, × 31,000; **B**, × 38,000) (with printing reduction of 13%)

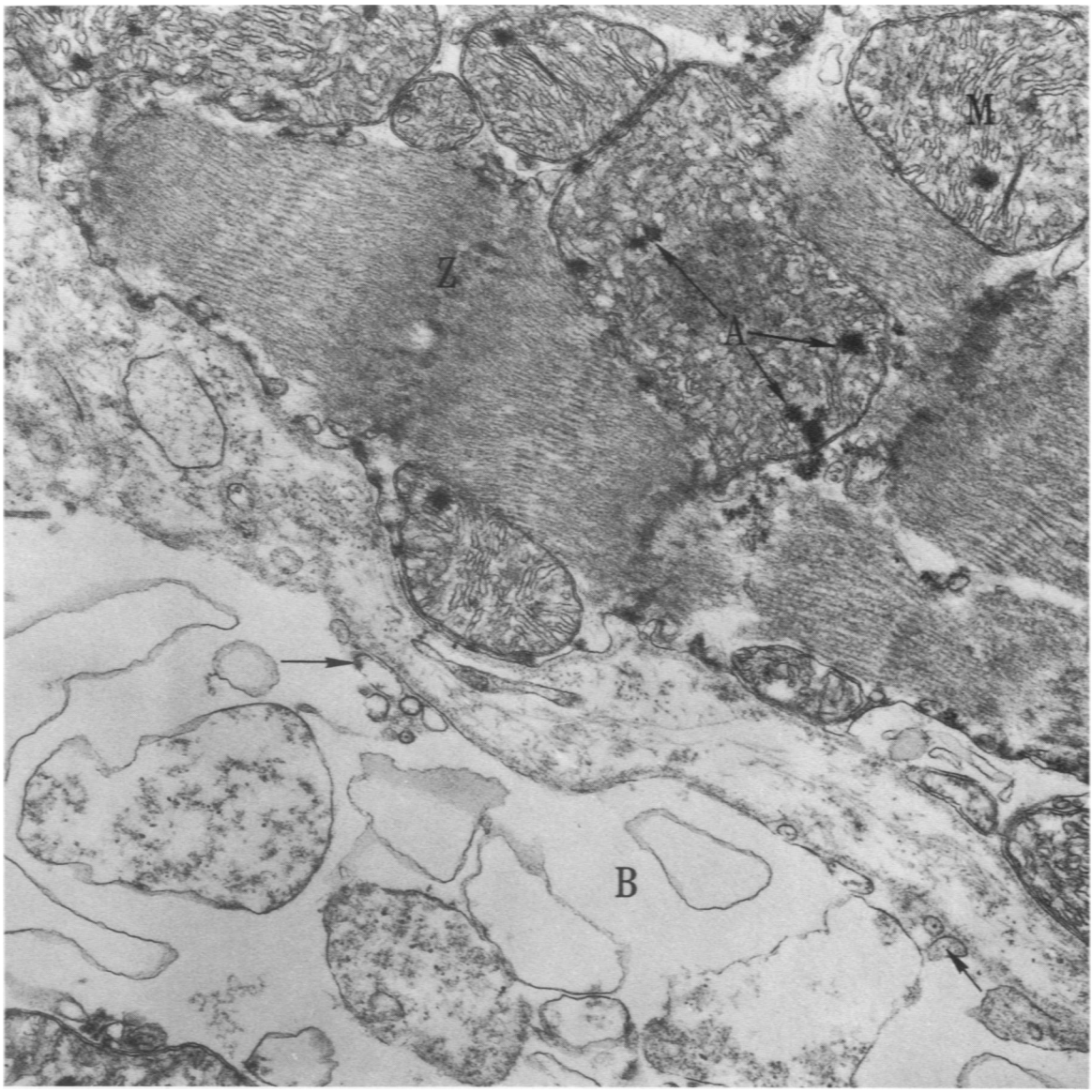


Figure 4—Posterior papillary muscle slice after 40 minutes of *in vivo* ischemia and 60 minutes of incubation. Slice incubation caused characteristic changes in myofibrillar architecture, including disappearance of the I bands and widening of the Z band zone. The mitochondria (*M*) are swollen and contain amorphous matrix densities (*A*). The sarcolemma of a subsarcolemmal bleb (*B*) of an adjacent cell is disrupted. Circular profiles of plasma membrane (*arrows*) are present beneath the remaining glycocalyx (*G*). (× 31,000) (with printing reduction of 13%)

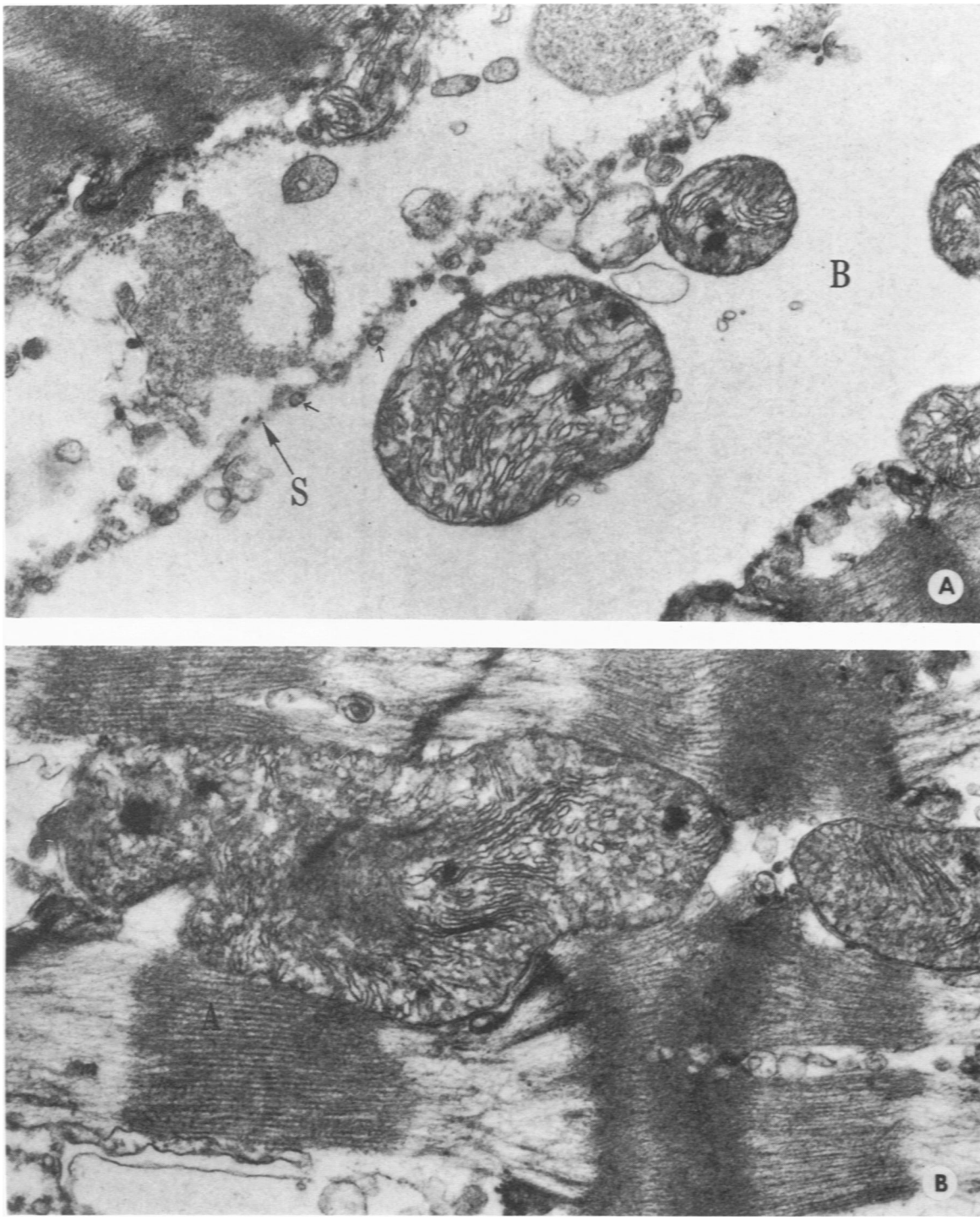


Figure 5—Posterior papillary muscle slice after 60 minutes of *in vivo* ischemia and 60 minutes of incubation. **A**—This shows a large subsarcolemmal bleb (*B*) containing two mitochondria, each of which contains large amorphous matrix densities. The remains of the sarcolemma (*S*) show glycocalyx and circular profiles of plasma membrane (*arrows*). **B**—Foci of hypercontraction of myofibrils are shown here. Two Z lines appear to be included in the aggregate at *lower right*, while an isolated A band (*A*) is seen at *lower left* between two superstretched I bands. A large mitochondrion contains several amorphous matrix densities. (**A** and **B**, × 28,000) (with printing reduction of 13%)